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Full Length Research Paper

Purification and characterization of a novel 1,3-propanediol oxidoreductase from *Klebsiella oxytoca*

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A novel 1,3-propanediol oxidoreductase (YqhD-1) found in *Klebsiella oxytoca* M5al was purified to homogeneity with a his-tag on a Ni-NTA column. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified protein showed a molecular weight of 42 kDa. When YqhD-1 was tested as a dehydrogenase, the optimal pH was 11 and the optimal temperature was 37°C. Both Zn^{2+} and Fe^{2+} promoted YqhD-1 activity, whereas Mn^{2+}, Ni^{2+}, SO_4^{2-} and EDTA markedly inhibited YqhD-1 activity. Broad substrate specificity was observed for YqhD-1 with activity for several alcohols, including 1,3-propanediol, 1,2-propanediol, propanol, glycerol, 1-butanol, 1,3-butanediol and isopentanol. However, the enzyme preferred high carbon number alcohols such as 1-butanol and isopentanol. When tested as an aldehyde reductase, YqhD-1 could convert acetaldehyde and propaldehyde to their respective alcohols. When 1,3-PD and propaldehyde served as substrates, the apparent Km values of the enzyme for NADP and NADPH were 0.14 and 0.64 mM, respectively.

Key words: *Klebsiella oxytoca, 1,3-propanediol oxidoreductase, purification, YqhD.*

INTRODUCTION

Some bacteria in *Klebsiella* genera could use glycerol as sole substrate to produce 1,3-propanediol (1,3-PD), which is an important chemical used in the synthesis of polytrimethylene terephthalate (PTT) (Celinska, 2010). In *Klebsiella pneumoniae*, glycerol is firstly to be dehydrated to 3-hydroxypropionaldehyde (3-HPA) by a B_{12}-dependent glycerol dehydratase, which is then reduced to 1,3-PD by an NADH-linked 1,3-PD oxido-reductase (PDOR).

Recently, a non-specific *Escherichia coli* alcohol dehydrogenase, YqhD, has attracted considerable attention. The enzyme has a function similar to PDOR, which catalyzes the conversion of 3-HPA into 1,3-PD, although, it shares little amino acid identity with PDOR DuPont published the first report on *E. coli* YqhD, and they used it instead of PDOR to produce 1,3-PD, thereby obtaining the highest yield of 1,3-PD to date (Nakamura and Whited, 2003). Further study showed that *E. coli* YqhD was both an NADP-dependent alcohol dehydro-genase (Sulzenbacher et al., 2004) and an NADPH-dependent aldehyde/furfural reductase (Perez et al., 2008; Miller et al., 2009).

In a previous study, it was found that Klebsiella *oxytoca* did not have a typical PDOR but only an uncharacterized oxidoreductase similar to *E. coli* YqhD (87% amino acid sequence identity), which reduces 3-HPA to 1,3-PD (Yang et al., unpublished). This is different from *K. pneumoniae*, which was shown to have both an *E. coli* YqhD homologue and PDOR (Seo et al., 2010). In order to differentiate the *K. oxytoca* YqhD from the *E. coli* and *K.pneumoniae* YqhD proteins, it was designated YqhD-1.

Thus far, the studies on YqhD mostly focused on the

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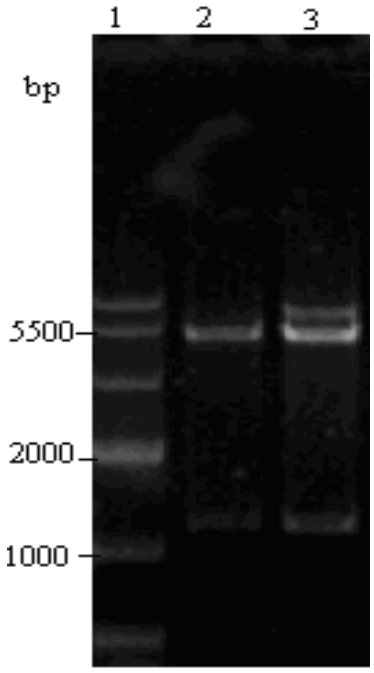

Figure 1. Enzyme analysis of recombinant plasmids constructions by agarose gel electrophoresis. Lane 1: DNA Marker; Lane 2: pHY *Nco*I and *Hin*DIII restriction endonuclease digestion; Lane 3: pYH *Nco*I and *Hin*DIII restriction endonuclease digestion.

construction of engineered strains to yield 1,3-PD using the *E. coli yqhD* gene (Zhu et al., 2009; Zhuge et al., 2010; Tang et al., 2009). There is no available report on the properties of YqhD-1 in *K. oxytoca*. In the present study, we reported for the first time the purification and characterization of the novel 1,3-PD oxidoreductase (YqhD-1) from *K. oxytoca*, which is the basis for investigation of the genetic diversity of *yqhD* genes among different 1,3-PD-producing bacteria and *E. coli*.

MATERIALS AND METHODS

Organisms, plasmids and growth conditions

K. oxytoca M5al, *E. coli* DH5α and *E. coli* BL21(DE3) were preserved in our laboratory. The pMD18-T Simple vector and pET28a were purchased from Takara (Japan) and Novagen (Wisconsin, USA), respectively. *E. coli* BL21(DE3) was grown aerobically at 37°C in LB medium containing 50 μg/ml kanamycin.

Construction of recombinant plasmids and strains

The *yqhD-1* genes with N-terminal and C-terminal his-tags were amplified by polymerase chain reaction (PCR) from *K. oxytoca* genomic DNA as a template using primer pairs HY1 (gtccatgggacaccaccaccaccaccacgccatgaataatttcgacctac, *Nco*I), HY2 (acgaagcttcggagaggaggttgattagc, *Hin*DIII) and YH1 (gtccatgggattgagggcaccagccatg, *Nco*I), YH2 (acgaagctttagtggtggtggtggtggcgcgcagcctcgtaaat, *Hin*DIII), respectively. The PCR products were purified and ligated into the pMD18-T Simple vector, resulting in plasmids pTHY and pTYH; these plasmids were then digested with *Nco*I and *Hin*DIII and ligated into the expression vector pET28a to generate recombinant plasmids pHY and pYH, respectively. These two recombinant plasmids were transformed into competent *E. coli* BL21(DE3) cells, and two recombinant strains, *E. coli* HY (expressing YqhD-1 with an N-terminal his-tag) and *E. coli* YH (expressing YqhD-1 with a C-terminal his-tag), were finally obtained. The recombinant plasmids were subjected to enzyme digestion and sequencing to verify the correct construction.

Expression of *yqhD-1* and purification of YqhD-1

When the cells expressing YqhD-1 grown in LB medium reached an optical density at 600 nm of 0.5, IPTG was added to a final concentration of 0.5 mM and was incubated for 7 h. The cells were centrifuged at 12,000 ×*g* for 10 min at 4°C, and after washing with lysis buffer (50 mM sodium phosphate, pH 8.0; 500 mM NaCl; 10 mM imidazole), cells were resuspended in the same buffer (10 mL). Crude cell extracts were prepared by ultrasonic treatment for 10 min (Power 240 W). After centrifugation (12,000 ×*g* for 25 min at 4°C), the supernatant was used for purification.

The supernatant (8 ml) was loaded onto a nickel nitrilotriacetic acid-agarose (Ni-NTA) affinity chromatography column, which was pre-equilibrated with washing buffer (50 mM sodium phosphate, pH 8.0; 500 mM NaCl; 20 mM imidazole). After washing with 20 column volumes of the same buffer, the enzyme was eluted with 2.5 ml of elution buffer (50 mM sodium phosphate, pH 8.0; 500 mM NaCl; 50 to 250 mM imidazole).

The eluted fractions were analyzed using SDS-PAGE (10%). The proteins on the gel were stained with Coomassie brilliant blue R-250. Marker proteins with molecular weights ranging from 14.4 to 94.0 kDa (14.4, 20, 26, 33, 45, 66.2, 94 kDa) were used to estimate the molecular weights of the expression products.

Enzyme assays

Enzyme activity was calculated from the linear slope of increasing or decreasing absorption of NADPH at 340 nm (Johnson and Lin, 1987; Perez et al., 2008.) at room temperature. One unit of enzyme activity was defined as the amount of protein that produces or consumes 1 nmol of NADPH per minute. Unless otherwise stated, the reaction mixture contained 100 mM potassium carbonate (K_2CO_3) buffer (pH 9.0), 100 mM 1,3-PD, 1 mM NADP, 30 mM $(NH_4)_2SO_4$ and 40 μg/ml purified YqhD-1. When YqhD-1 was tested as an aldehyde reductase, potassium phosphate buffer (pH 7.0), was used and the substrates were replaced with aldehydes and NADPH. The total protein concentration was determined by the method of Bradford using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Construction of recombinant plasmids

Two target products of *yqhD-1* genes were amplied by PCR using the primer pairs HY1, 2 and YH1, 2 from the template *K. oxytoca* M5al genome. Finally, two target recombinant plasmids were obtained by ligating the products into the *Nco*I and *Hin*DIII sites of the plasmid pET28a (Figure 1), which were further proved by

Table 1. Purification steps of the YqhD-1.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	4.99	754.3	151.2	1	100
Ni-NTA agarose	3.18	638.6	200.8	1.33	84.7

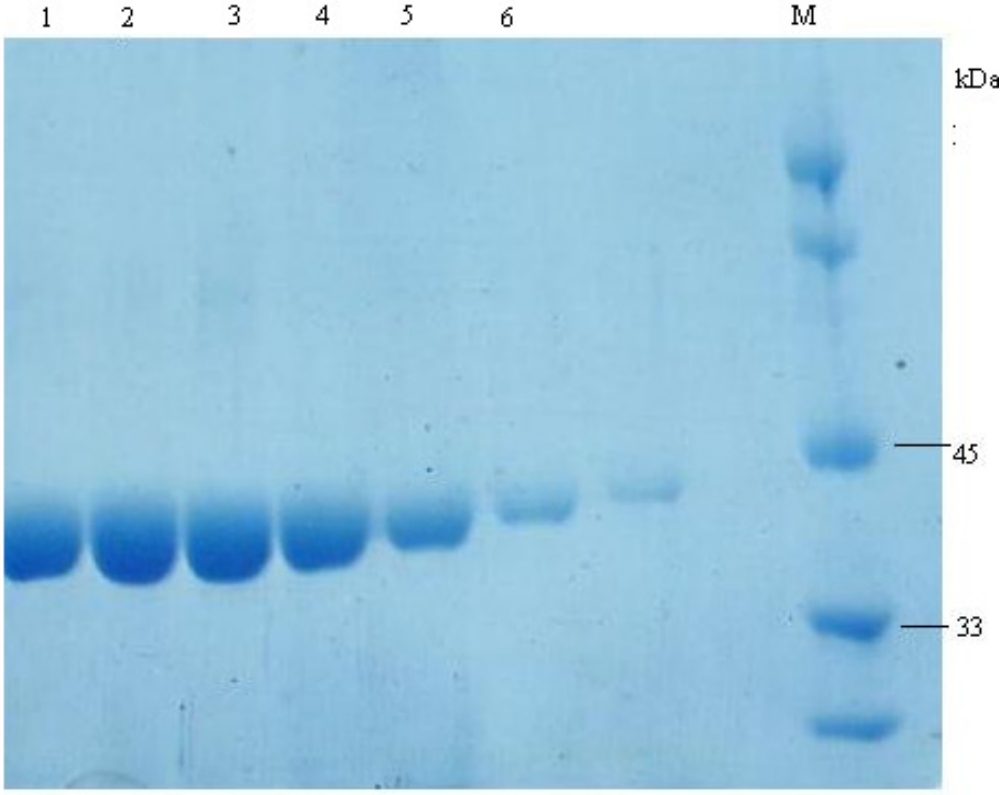

Figure 2. SDS-PAGE of purified YqhD-1. Lane 1, Elution with 50 mM elution buffer; lane 2, elution with 100 mM elution buffer; lane 3, elution with 70 mM elution buffer; lane 4, elution with 150 mM elution buffer; lane 5, elution with 200 mM elution buffer; lane 6, elution with 250 mM elution buffer; M, molecular weight markers (26, 33, 45, 66.2, 94 kDa). Elution buffer (50 mM sodium phosphate, pH 8.0; 500 mM NaCl; 50 to 250 mM imidazole).

sequencing (GenBank accession no: EF683586). The constructs were transformed into *E. coli* BL21(DE3) for recombinant YqhD overexpression.

Expression of *yqhD* in *E. coli* and purification of YqhD-1

His-tagged YqhD-1 (with an N-terminal his-tag) was expressed and purified as described in materials and methods. It was purified 1.33-fold with an overall yield of 84.7% and specific activity of 200.8 U/mg (Table 1). The results show that the soluble protein was in the cell-free crude extract of *E. coli* HY, and that no such soluble protein existed in the cell-free crude extract of *E. coli* YH (data not shown), which is similar to what was found with glutamine synthetase (Li et al., 2009).

The purity of the enzyme was examined using SDS-PAGE. As shown in Figure 2, the purified enzyme appeared to be homogeneous. Its molecular weight (MW) was approximately 42 kDa, which was identical to the predicted MW of the *yqhD* gene. Because the deduced gene product of *yqhD-1* has 87% identity with *E. coli* YqhD, the enzyme could also be a tetramer of a 42 kDa polypeptide (Sulzenbacher et al., 2004; Zhu et al., 2009).

The YqhD-1 amino acid sequence (EF683586) has 87.34 and 93.28% similarity with the *E. coli* YqhD sequence (NC_004431. 1) and *K. pneumoniae* AS 1.1736 YqhD sequence (EU012494), respectively. Additionally, there are no similarity between amino acid

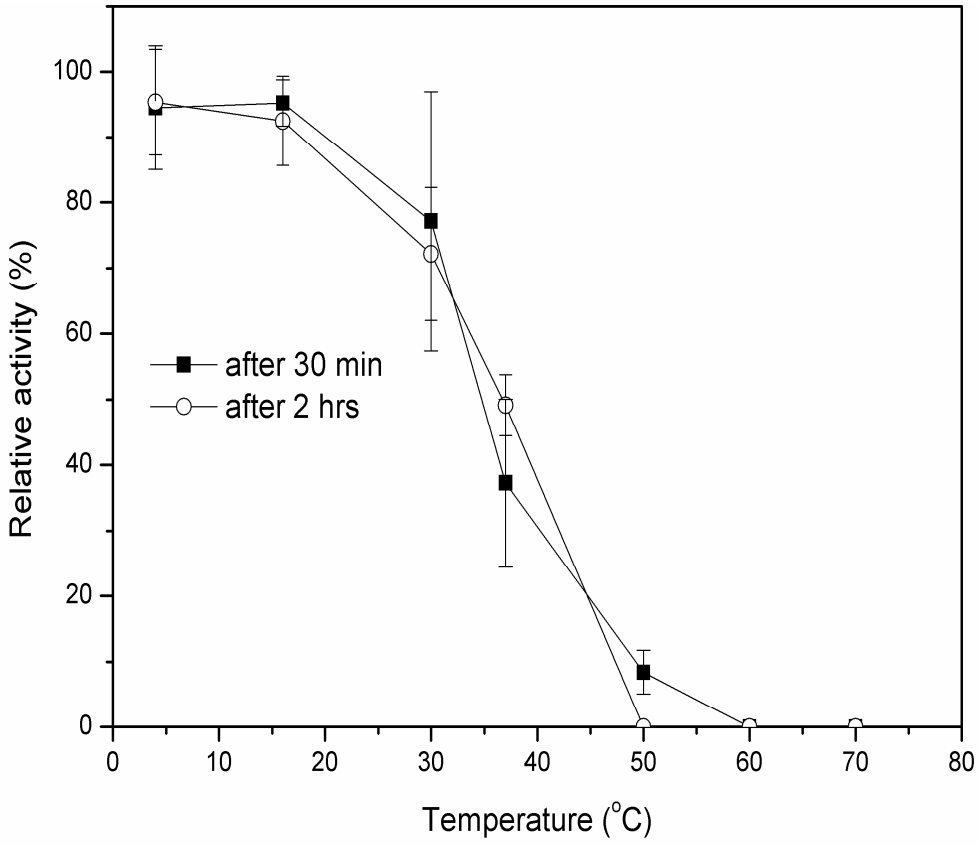

Figure 3. Temperature stability of YqhD-1. Temperature stability was determined by pre-incubating purified YqhD-1 in 100 mM K_2CO_3 buffer (pH 9.0) at 4, 16, 30, 37, 50, 60 and 70°C for 0.5 and 2 h. Enzyme activity was expressed as a percentage relative to the maximum value. All the experiments were repeated at least 3 times.

sequences of the YqhD-1 and typical 1,3-PD oxido-reductase (PDOR, encoded by the gene *dhaT*).

Effect of temperature and pH on YqhD-1 activity

As shown in Figure 3, YqhD-1 was stable at 4 and 16°C, and it retained 51% activity after incubation at 37°C for 2 h. The optimal temperature for activity was 37°C and the optimal pH was 11 (Figures 4 and 5).

Effect of monovalent and divalent cations, NH_4^+, SO_4^{2-} and EDTA on YqhD-1 activity

The influence of metal ions, NH_4^+ and SO_4^{2-} on YqhD-1 was investigated and the results are shown in Table 2. The relative activity of YqhD-1 increased 8-, 3.5- and 2-fold with addition of Zn^{2+}, Fe^{2+} and Co^{2+}, respectively suggesting that these ions are beneficial for the enzyme. This was somewhat similar to what was observed for the enzymes from *E. coli* and *Pyrococcus furiosus*, which required Zn^{2+} for full activity (Ma and Adams, 1999).

The addition of Ca^{2+}, Cu^{2+} or NH_4^+ did not affect YqhD-1 activity. However, YqhD-1 activity was inhibited by Mn^{2+} and Ni^{2+}. In contrast, the 1,3-PD dehydrogenases from *K. pneumoniae* (Johnson and Lin, 1987), *Lactobacillus buchneri*, *Lactobacillus brevis* (Veigadacunha and Foster, 1992), *Citrobacter freundii* (Daniel et al., 1995) and *Clostridium butyricum* (Malaoui and Marczak, 2000) required either Mn^{2+} or Fe^{2+} for full activity.

When $(NH_4)_2SO_4$ was added to the reaction mixture, more than 40% of the activity was lost. Further study showed that SO_4^{2-}, but not NH_4^+, was the inhibitor of YqhD-1 activity. This was different from what was observed for the 1,3-PD dehydrogenase of *C. butyricum* E5 mutant D, for which NH_4^+ was shown to be the most effective activator (Malaoui and Marczak, 2001).

When EDTA was added to the reaction, the relative activity of YqhD-1 decreased by 89.7% which showed that YqhD-1 requires metal ion as a cofactor (Table 2).

Kinetic constants for substrates

YqhD-1 was preferentially active with NADP or NADPH,

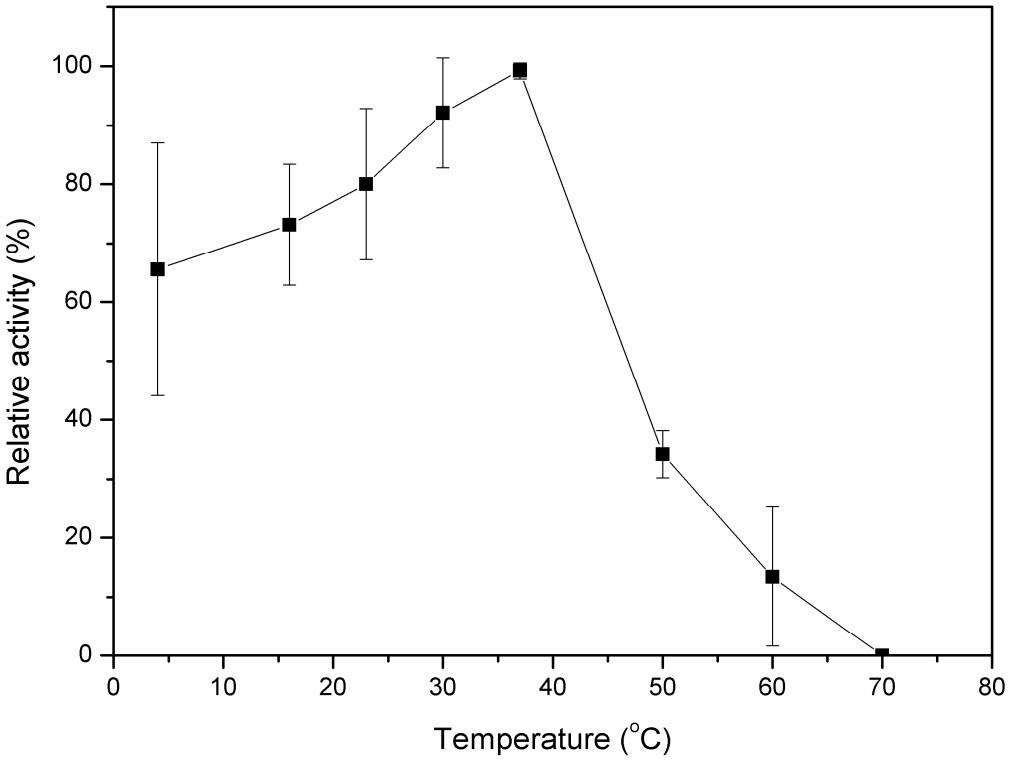

Figure 4. Optimal temperature of YqhD-1. Assays to determine the optimum reaction temperature were performed over a range of 4 to 50°C at pH 9.0. Enzyme activity was expressed as a percentage relative to the maximum value. All the experiments were repeated at least 3 times.

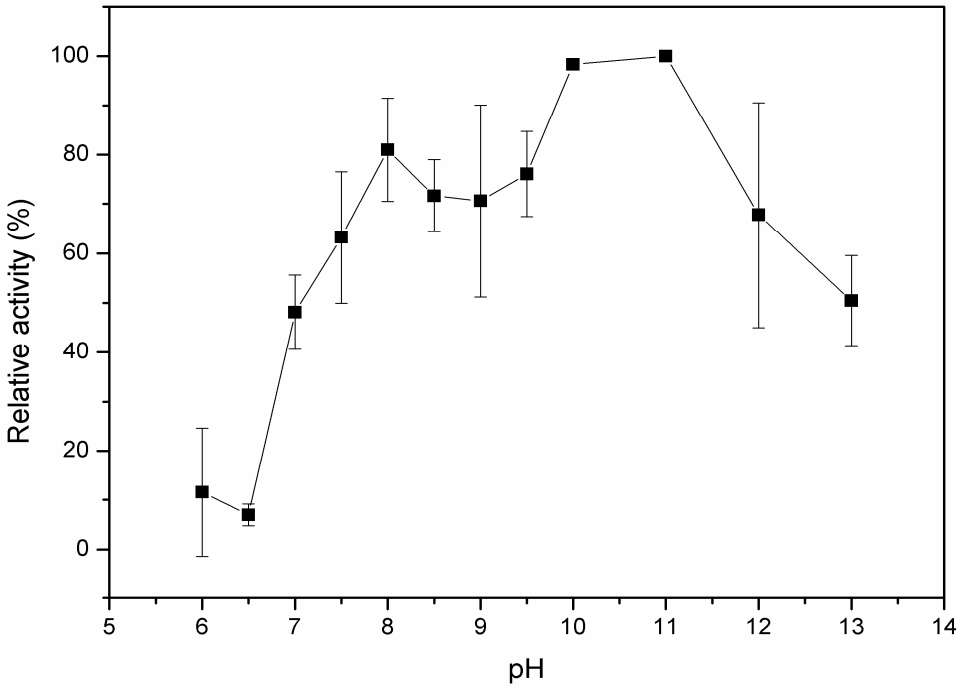

Figure 5. Optimal pH of YqhD-1. To determine the optimal pH, the experiments were run in 100 mM K_2CO_3 buffer adjusted to the appropriate pH with either 3 M KOH or 3 M HCl. Enzyme activity was expressed as a percentage relative to the maximum value. All the experiments were repeated at least 3 times.

Table 2. The effect of monovalent and divalent cations, NH_4^+, SO_4^{2-} and EDTA on YqhD-1 activity.

Additive	Concentration (mM)	Relative activity (%)
None		12.6
Zn^{2+}	1	100
Fe^{2+}	1	56.8
Co^{2+}	1	37.8
Mg^{2+}	1	14.3
Cu^{2+}	1	10.8
Ca^{2+}	1	7.1
Ni^{2+}	1	4.6
Mn^{2+}	1	0
NH_4^+	30	12.4
SO_4^{2-}	5	7.2
EDTA	1	1.3

YqhD-1 was incubated with metal ions, NH_4^+, SO_4^{2-} or EDTA in 100 mM K_2CO_3 buffer (pH 9.0) along with 100 mM 1,3-PD and 1 mM NADP to determine their effects on activity. Enzyme activity was expressed as a percentage relative to the activity determined in the presence of Zn^{2+}. All experiments were repeated at least 3 times.

Table 3. The apparent Km values of YqhD-1 for substrates and coenzymes.

Alcohol	Km (mM)	Aldehyde	Km (mM)	Coenzyme	Km (mM)
Methanol	ND	Formaldehyde	ND	NADP	0.14
Ethanol	690.8	Acetaldehyde	620.8	NADPH	0.64
Ethanediol	ND	Propaldehyde	529.8		
1,3-Propanediol	94				
1,2-Propanediol	2769.5				
Propanol	1244.4				
Isopropanol	ND				
Glycerol	6210.8				
1-Butanol	31.1				
1,3-Butanediol	136.2				
Isopentanol	18.6				

The apparent Km values of YqhD-1 were determined from the results of experiments in which a fixed concentration of the substrate or coenzyme was used together with an appropriate concentration range of the other reactants. The Km values were calculated by nonlinear regression to the Michaelis-Menten equation from Lineweaver-Burk plots. $(NH_4)_2SO_4$ was omitted from the assay mixtures for the determination of Km values. ND, not detected. All experiments were repeated at least 3 times.

and was much less active with NAD or NADH (data not shown). However, YqhD-1 was not highly substrate specific, since it was able to oxidize a broad range of alcohols, including ethanol, 1,3-propanediol, 1,2-propanediol, propanol, glycerol, 1-butanol, 1,3-butanediol and isopentanol. Further, it can reduce acetaldehyde and propaldehyde to their corresponding alcohols (Table 3). This observation is consistent with the findings for YqhD from *E. coli* and 1,3-PD dehydrogenase from *L. buchneri* and *L. brevis* (Veigadacunha and Foster, 1992), but dissimilar to the typical PDOR from *K. pneumoniae*, which only catalyzes the reaction of 1,3-PD to 3-HPA when tested as a dehydrogenase.

As shown in Table 3, YqhD-1 exhibited Michaelis-Menten kinetics, and the apparent Km values for ethanol, 1,3-propanediol, 1-butanol and isopentanol were 690.8, 94, 31.1 and 18.6 mM, respectively. This indicated that the affinity of YqhD-1 for isopentanol, 1-butanol and 1,3-propanediol was higher than for ethanol. This result was similar to that described for *E. coli* YqhD as an alcohol dehydrogenase, which has a preference for alcohols longer than C_3 (Sulzenbacher et al., 2004). It was notable that YqhD-1 activity was not detected using methanol as a substrate.

In addition, the presence of more alcoholic hydroxyls in substrates with the same carbon atoms led to a decrease in YqhD-1 specificity, for example, ethanol and ethanediol, 1,3-PD and glycerol, and 1-butanol and 1,3-butanediol. This interesting phenomenon is different from what was observed with the 1,3-PD dehydrogenases from *K. pneumoniae* (Johnson and Lin, 1987), *C. butyricum* (Malaoui and Marczak, 2000), *C. freundii* (Daniel et al., 1995) and *Lactobacillus reuteri* (Talarico, 1990). The 1,3-PD dehydrogenase of *K. pneumoniae* and *C. butyricum* were very specific for 1,3-PD, whereas the *C. freundii* enzyme preferentially oxidized substrates containing two primary alcohol groups separated by one or two carbon atoms (such as 1,3-PD and 1,4-BD), and the *L. reuteri* enzyme exhibited the highest activity for substrates that have adjacent hydroxyl functionality such as glycerol and 1,2-PD.

The apparent Km values for acetaldehyde and propaldehyde were 620.8 and 529.8 mM, respectively suggesting that YqhD-1 preferred propaldehyde, which is consistent with the finding that *E. coli* YqhD exhibited aldehyde reductase activity (Perez et al., 2008).

When propaldehyde served as a substrate, the apparent Km value of YqhD-1 for NADPH was 0.64 mM; when 1,3-PD was the substrate, the Km value of YqhD-1 for NADP was 0.14 mM, which is similar to that of *E. coli* YqhD (0.15 mM) (Zhu et al., 2009). This low Km value means that YqhD-1 has a high affinity for NADP in the reaction from 3-HPA to 1,3-PD, in contrast, the Km value of the 1,3-PD dehydrogenase encoded by the *dhaT* gene from *K. pneumoniae* for NAD is 2-fold higher (0.31 mM) (Johnson and Lin, 1987).

Conclusions

A novel 1,3-PD oxidoreductase from *K. oxytoca* (YqhD-1) was purified with a his-tag using a Ni-NTA agarose column, and its molecular weight is approximately 42 kDa. This enzyme can catalyze the conversion of 3-HPA to 1,3-PD, which is similar to PDOR from *K. pneumoniae*. However, YqhD-1 showed characteristics different from the *K. pneumoniae* PDOR, and had properties similar to *E. coli* YqhD. This is the basis for the investigation of the genetic diversity of *yqhD* gene among different 1,3-PD-producing bacteria and *E. coli*.

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